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ABSTRACT

Antimicrobial disk susceptibility tests serve as standard assays for measuring the activity of compounds against pathogenic bacteria. In the current study, two plant-derived proprietary essential oil blends were tested for their antibacterial activity against five common strains of pathogenic bacteria using disk susceptibility tests. A formulation intended for topical use (EOF 1) inhibited the growth of *Escherichia coli, Klebsiella pneumoniae,* and *Staphylococcus aureus* as evidenced by zone inhibition diameter measurements when compared to those reported for standard antibiotics. EOF 1 exhibited no activity against *Proteus vulgaris* and *Staphylococcus epidermidis.* The second formulation (EOF 2), intended for inhalation use, inhibited the growth of all five test bacteria strains with zone inhibition diameters two to three times greater than those reported for standard antibiotics. The growth of all five bacteria strains was inhibited when a cotton swab impregnated with EOF 2 was suspended above the bacterial lawn, indicating a true vapor or fume effect by this formulation.

Key words: essential oils, antibacterials, disk susceptibility testing, zones of inhibition, aromatherapy, botanical oil extracts, antimicrobials.

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INTRODUCTION

The antimicrobial properties of some plant-derived essential oils have been recognized for hundreds of years[1,2] and have been documented in scientific studies.[3-7] It has been demonstrated that the antimicrobial activities of one natural oil, tea tree oil, obtained from *Melaleuca alternifolia,* are attributable to its hydrocarbon and terpine constituents, including terpinen-4ol, α-terpineol and linalool.[8]

The purpose of this study was to determine the inhibitory effect of two botanical combinations of essential oils against five common and clinically significant bacterial pathogens. Antimicrobial activity was assayed by the standard method adopted from the National Committee on Clinical Laboratory Standards (NCCLS) for antibiotic susceptibility testing.[9,10]

One of the two proprietary formulations (EOF 1) was developed for topical use and the other (EOF 2) for inhalation. Besides the direct contact disk sensitivity method we devised a technique for the inhalation formulation where a cotton swab was suspended above the agar-based bacterial lawn, mimicking a true vapor or aroma effect.

MATERIALS AND METHODS

Culture Preparation

The following strains of gram negative and gram positive bacteria were purchased from American Type Culture Collection (ATCC) Manassas, VA: *Escherichia coli* ATCC,® 25922, *Klebsiella pneumoniae* ATCC,® 27736, *Staphylococcus aureus* ATCC,® 25923, *Proteus vulgaris* ATCC,® 5380, and *Staphylococcus epidermidis* ATCC,® 12228. Each strain was plated out on blood agar plates and incubated for 18 hours at 35°C. Three to five identical

colonies from each agar plate were lifted with a sterile wire loop and transferred into a tube containing 5 mL of tryptic soy broth (TSB). Turbidity of each bacterial suspension was adjusted with TSB media to reach an optical comparison to that of a 0.5 McFarland standard, resulting in a suspension containing approximately 1 to 2 x 10^8 CFU/mL. A Wickerham Card (Hardy Diagnostics, Santa Maria, CA) was used for the visual comparison.[11]

Within 15 minutes after adjusting the turbidity of the inoculum suspension, Mueller-Hinton agar plates were inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure even distribution of the inoculum. As a final step, the rim of the agar was also swabbed.

Susceptibility Disk Method

The essential oil formulations were acquired from Lane Labs, Allendale, NJ, manufactured and formulated by Bio Excel, Sonoma, CA. Both formulas contain a combination of plant-derived oil extracts (see Table 1). Fifteen microliters of either tryptic soy broth (Control), or EOF 1, or EOF 2 was placed on separate 0.25-inch blank filter paper disks (Hardy Diagnostics, Santa Maria, CA.) The disks were dispensed onto the surface of the inoculated agar plates and incubated at 35°C for 18 hours. The diameters of the zones of complete inhibition were recorded and each Petri dish were photographed.

Suspended Cotton Swab Method

Sterile cotton swabs were dipped in either sterile water for control plates or in EOF 2 and pressed firmly against the sides of the vessels to prevent dripping. They were then taped to the lid of each Petri dish. The swabs were suspended above the agar to avoid contact with its surface so that only the volatile components of EOF 2 elicited inhibitory activity.

Table 1. Essential Oil Formulation Ingredients.

EOF 1 *Melaleuca alterniflolia* *Cymbopogon martinii* *Commiphora molmol* *Thymus vulgaris linalool* *Helichrysum itallicum serot* Vitamin E
EOF 2 *Melaleuca alternifloria* *Ravensara aromatica* *Rosmarinus officinalis camphora* *Radiata* *Thymus vulgaris thymol* *Lavandula vera* *Menta piperita*

RESULTS

The zones of bacterial inhibition were measured to the nearest whole millimeter. The results of susceptibility disk tests showed moderate zone inhibition with three of the five bacterial strains in the EOF 1-treated cultures. Inhibition diameters in this group ranged from 11 to 18 mm. All five bacterial cultures treated with EOF 2 exhibited appreciable increases of zones of inhibition ranging from 25–68 mm. Both groups resulted in irregular zone patterns, unlike those typically seen in standard antibiotic susceptibility tests. Antibiotics, for the most part, present a clean circular edge within the bacterial lawn, whereas the essential oil formulations produced an asymmetric jagged or spiked edge (see Fig 1). This effect may be a result of the pattern of radial diffusion penetration through troughs of uneven growth distribution within the bacterial lawn, giving rise to micro channels of less dense or sparse bacterial accumulation.

The suspended swab method, incorporating EOF 2, resulted in zones of inhibition ranging from 26–36 mm in diameter in all five bacterial strains studied. An elliptical pattern mimicking the shape of the cotton impregnated with EOF 2 was observed (see Fig. 2). There appeared to be a concentration gradient of clear pronounced inhibition in the center, gradually feathering and fading to the periphery of the zone in four of the five bacterial strains tested. Presumably, the highest concentration of vapor molecules was at the center of the swab. This feathered zone edge effect was not prominent with *Staphylococcus aureus*.

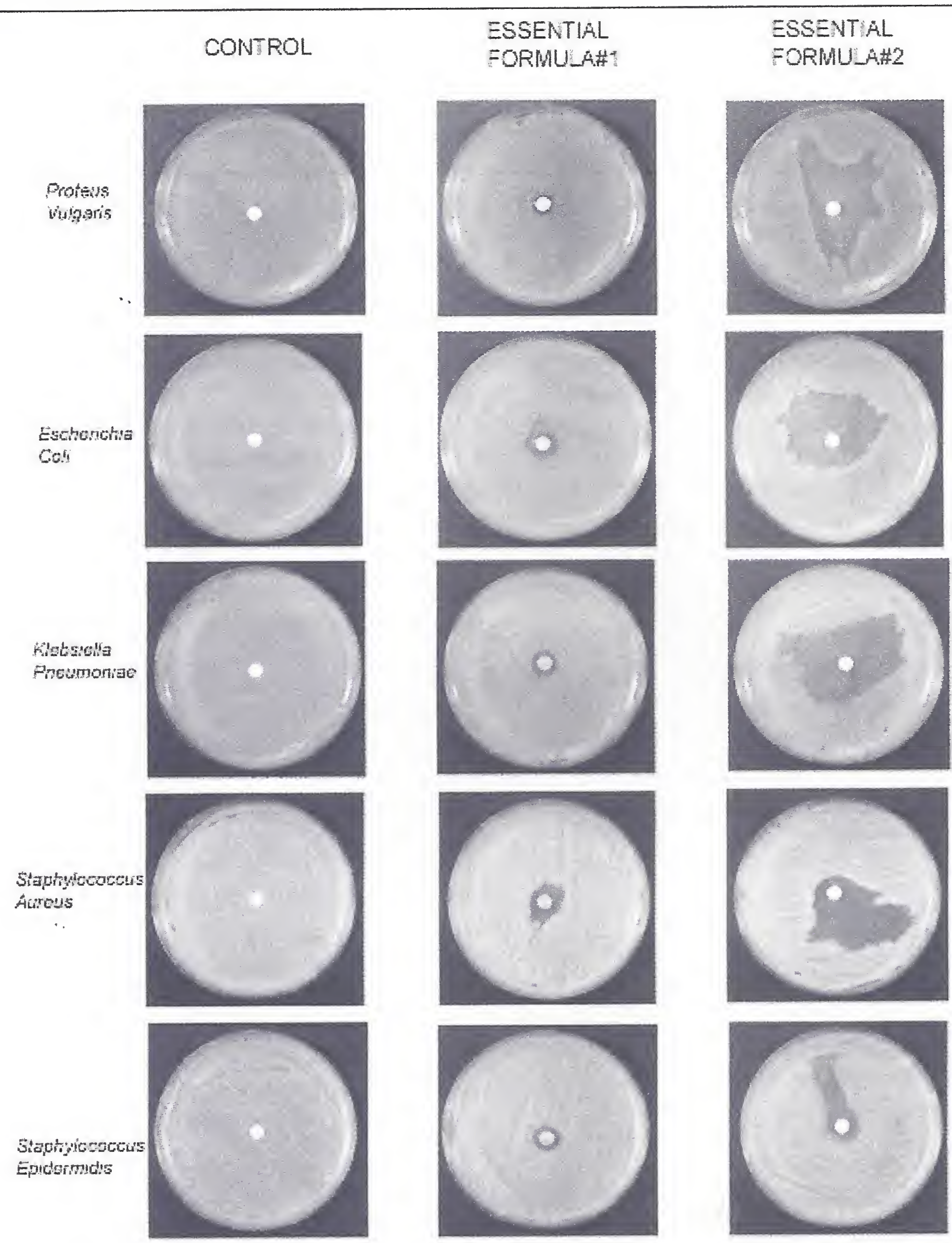
DISCUSSION

The range of sensitivity to a wide variety of antibiotics by strains of bacteria similar to those used in this study (13 – 29 mm with concentrations of antimicrobials ranging from 1 μg to 350 μg per disk content) is documented in the NCCLS "tables of zone diameter interpretive standards and equivalent minimal inhibitory concentration (MIC) breakpoints."[9]

In this screening study, the bacteriostatic and/or bactericidal activities of two combination essential oil formulations (EOF 1, EOF 2) were observed against five ubiquitous strains of both gram negative and gram positive pathogenic bacteria. EOF 1 is intended for topical use and showed minimal to moderate antibacterial action comparable to the lower zone inhibition ranges reported for most antibiotics, 11–18 mm versus 13–29 mm respectively. *Escherichia coli, Klebsiella pneumoniae* and *Staphylococcus aureus* were more sensitive to EOF 1 as evidenced by 15 mm, 16 mm, and 18 mm inhibited zone diameters respectively. Insensitivity was observed with *Proteous vulgaris* and *Staphylococcus epidermidis* with 11mm and 12mm zone diameters respectively, when compared to NCCLS-reported antibiotic minimal inhibitory range diameters.

EOF 2 is targeted for inhalation use. When the disk

Figure 1. Susceptibility of Bacteria to Essential Oils

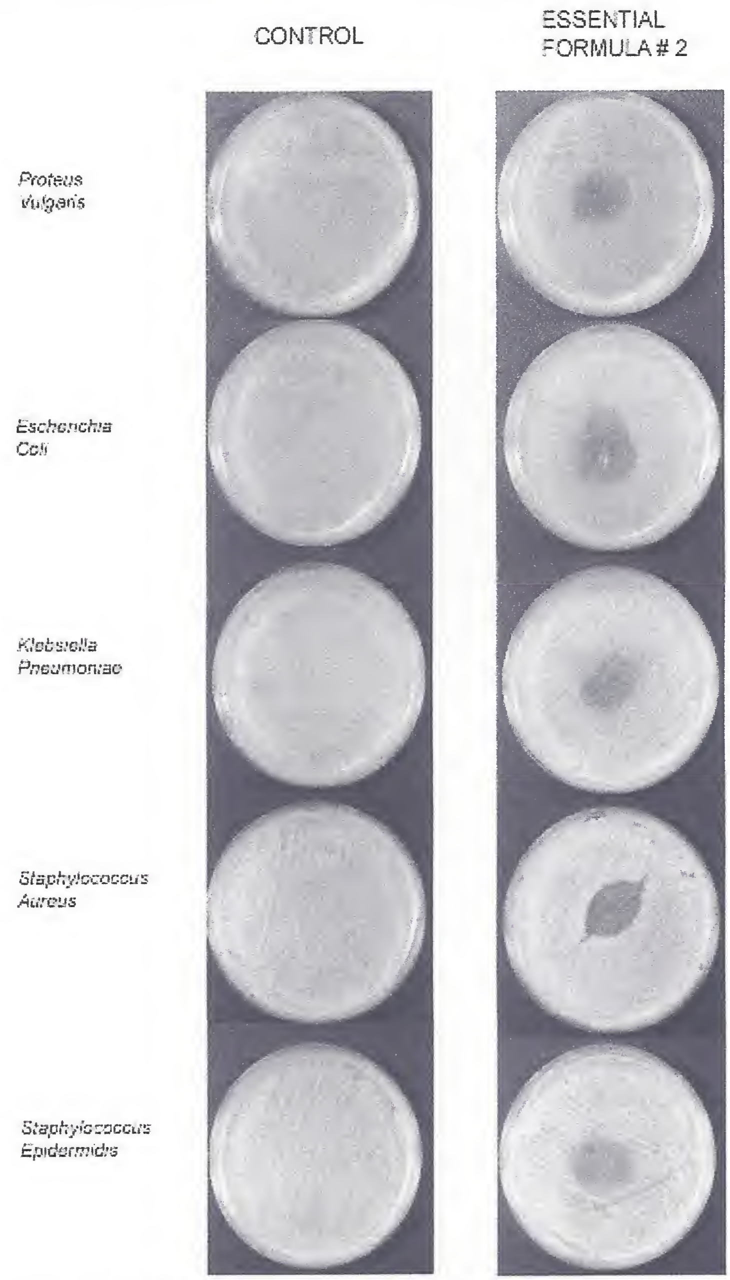


Each 8 mm sensitivity disk was impregnated with 15 μ l of either sterile TSB media for controls or 15 μ l of each essential oil formula.

method was utilized, the inhibition zone diameters for all bacterial strains were approximately two to three times the diameter of those of NCCLS-reported antibiotic sensitivity tests, 25–68 mm compared to 13–29 mm respectively. The suspended swab method more closely paralleled the intended inhalation use of EOF 2 by permitting only the gaseous phase of the formulation to make contact with the bacteria hence giving meaning to so called, aromatherapy. The inhibition seen by EOF 2 is greater than twice the reported antibiotic inhibitory diameters (26–36 mm) and is noteworthy since only a small concentration of the vapor molecules would probably come in contact with the growing bacteria. The inhalation application of EOF 2 may prove useful in the prevention and/or treatment of upper respiratory infections caused by some strains of bacteria, or in the treatment of viral-induced secondary bacterial infections.

With the increase of worldwide bacterial resistance of many strains of disease-producing bacteria, there is a need

Figure 2. Bacteria Susceptibility to Essential Oils



Cotton swabs were dipped in either sterile water for control plates or dipped in Essential Oil Formula #2 and taped to the lid of each Petri dish. The swabs were suspended above the agar to avoid contact with its surface. The zones of inhibition were a result of the vapor from the essential oil.

to access new and complementary approaches to antibiotic therapy. This screening study in the laboratory indicates that essential oils may be considered to be used in combination with standard topical and antibiotic therapies. However, to verify clinical utility, it is necessary to extend this research to human applications against similar strains of pathogens and examine dose responses of both topical and inhalation forms of the oils as well as testing various modes of administration. Because of minimal, if any, toxicity[12] and pleasant odor, these oils may have the advantage of greater acceptance by patients and the community.

This initial antimicrobial screen also warrants further studies with these formulations on antibiotic-resistant strains and other pathogens such as viruses, fungi, mycoplasma, chlamydia, and yeasts.

Table 2. Bacterial Zone of Inhibition Diameters with Essential Oil Formulations

	Zone Diameter (mm)*	
Bacteria	**EOF 1**	**EOF 2**
Escherichia coli	15	45
Staphylococcus epidermidis	12	25
Staphylococcus aureus	18	47
Klebsiella pneumoniae	16	55
Proteous vulgaris	11	68

**nearest whole millimeter*

Table 3. Bacterial Zone of Inhibition Diameters with EOF 2 Suspended Swab Test

Bacteria	Zone Diameter (mm)*
Proteous vulgaris	26
Klebsiella pneumoniae	25
Escherichia coli	32
Staphylococcus epidermidis	25
Staphylococcus aureus	36

**nearest whole millimeter*

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